Appendix L-1

Outlier Characterization for the 3T3 and NHK NRU Test Methods with the RC Regression

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L.1 Discordant Results for the 3T3 and NHK NRU Test Methods and RC Millimole Regression

The RC millimole regression and each *in vitro* NRU test method were used to identify discordant results among the reference chemicals tested in the validation study (i.e., those for which the rodent LD_{50} was not accurately predicted by the *in vitro* NRU IC_{50}). Discordant chemicals are also referred to as outliers. Once identified, discordant chemicals were then evaluated for common characteristics that may assist in determining the types of chemicals that are not suited for use in the 3T3 and NHK NRU test methods to determine starting doses for acute systemic toxicity assays. **Sections L.1.1** and **L.1.2** identify discordant chemicals for the RC weight regression and the combined 3T3 or the combined NHK NRU weight regressions (see **Tables L1-1 and L1-2**). Discordant chemicals for the millimole regressions are discussed in this appendix.

L.1.1 Identification of Discordant Chemicals

For each *in vitro* NRU test method, predicted LD_{50} values for the reference chemicals were determined using the geometric mean IC_{50} of the three geometric mean laboratory values in the RC millimole regression. Discordant chemicals were identified using the RC method (Halle 1998): a difference greater than 0.699 (or log 5) for log observed LD_{50} (in mmol/kg) minus log predicted LD_{50} identifies a chemical as discordant (i.e., an outlier) (see **Appendix J-1** for the 3T3 NRU test method and **Appendix J-3** for the NHK NRU test method for the predicted LD_{50} values for each chemical). **Table L1-1** lists the discordant chemicals for the RC millimole regression

Table L1-1 Discordant Chemicals for the 3T3 and NHK NRU Test Methods and the RC Millimole Regression

	3T3 NRU	NHK NRU
	Acetaminophen (+)	
	Arsenic III trioxide (-)	*Arsenic III trioxide (-)*
		Aminopterin (-)
5-Aminosalicylic acid		5-Aminosalicylic acid (+)
Busulfan	**Busulfan (-)**	**Busulfan (-)**
Caffeine		Caffeine (-)

Table L1-1 Discordant Chemicals for the 3T3 and NHK NRU Test Methods and the RC Millimole Regression

	3T3 NRU	NHK NRU
Cycloheximide	**Cycloheximide (-)**	**Cycloheximide (-)**
Dibutyl phthalate	***Dibutyl phthalate (+)***	***Dibutyl phthalate (+)***
	Dichlorvos (-)	Dichlorvos (-)
	Diethyl phthalate (+)	***Diethyl phthalate (+)***
Digoxin	***Digoxin (-)***	
Disulfoton	***Disulfoton (-)***	***Disulfoton (-)***
	Endosulfan (-)	Endosulfan (-)
Epinephrine bitartrate	Epinephrine bitartrate (-)	Epinephrine bitartrate (-)
Ethanol	**Ethanol (+)**	**Ethanol (+)**
	Fenpropathrin (-)	*Fenpropathrin (-)*
	Hexachlorophene (-)	
Lindane	*Lindane (-)*	*Lindane (-)*
Mercury II chloride	Mercury II chloride (-)	Mercury II chloride (-)
		Methanol (+)
Nicotine	Nicotine (-)	Nicotine (-)
Paraquat	Paraquat (-)	Paraquat (-)
Parathion	***Parathion (-)***	***Parathion (-)***
Phenobarbital	Phenobarbital (-)	Phenobarbital (-)
Phenylthiourea	Phenylthiourea (-)	Phenylthiourea (-)
	Physostigmine (-)	Physostigmine (-)
Potassium cyanide	Potassium cyanide (-)	Potassium cyanide (-)
Propylparaben	Propylparaben (+)	Propylparaben (+)
	Sodium hypochlorite (+)	Sodium hypochlorite (+)
		Sodium oxalate (-)
	Sodium selenate (-)	Sodium selenate (-)
	Strychnine (-)	*Strychnine (-)*
Thallium I sulfate	Thallium I sulfate (-)	
Triethylenemelamine	Triethylenemelamine (-)	Triethylenemelamine (-)
1,1,1-Trichloroethane		
Verapamil HCl	**Verapamil HCl (-)**	***Verapamil HCl (-)***

[1]Log LD_{50} (mmol/kg) = 0.435 log IC_{50} (mM) + 0.625. Log LD50 (mmol/kg) for discordant chemicals are > 0.699 from the RC regression.

(-) - toxicity underpredicted by the *in vitro* assay (i.e., the LD_{50} value predicted by the IC_{50} is higher than the *in vivo* LD_{50} value); (+) - toxicity overpredicted by the model (i.e., the LD_{50} value predicted by the IC_{50} is lower than the rodent reference LD_{50} value).

Bolded chemicals have active metabolites *in vivo* (see **Appendix F-2**).

Chemicals that showed evidence of insolubility (i.e., precipitates) during testing (see **Table 5-8**) are identified by italics.

Table L1-1 shows a comparison of the 22 RC outlier chemicals tested (identified in **Table 3-2**) with the outliers identified when using the 3T3 and NHK NRU results with the RC regression. Using the RC method of identifying discordant chemicals (Halle 1998), there were 30 discordant chemicals for the 3T3 assay and 31 discordant chemicals for the NHK assay. For the 58 RC chemicals that were tested, both test methods confirmed the outlier status of 19 of the 22 RC outliers tested, but the chemicals not identified as outliers were somewhat different for the 3T3 and NHK assays. The 3T3 NRU cytotoxicity test method did not identify caffeine, 5-aminosalicylic acid and 1,1,1-trichloroethane as outliers and the NHK test method did not identify digoxin, thallium sulfate, and 1,1,1-trichloroethane as outliers. The 3T3 assay identified four chemicals as outliers that were not identified as outliers by the RC: arsenic trioxide; hexachlorophene; acetaminophen; and diethyl phthalate. The NHK assay identified five chemicals as outliers that were not identified as outliers by the RC: aminopterin; arsenic trioxide; sodium oxalate; diethyl phthalate; and methanol.

L.1.2 Evaluation of Discordant Chemicals

To determine the attributes that may be used in the future to identify chemicals that would result in discordant predictions, a number of physico-chemical characteristics were evaluated for their frequency of occurrence among the discordant chemicals versus the entire set of test chemicals. This section provides a summary of these analyses for the discordant chemicals identified among the validation set based on the RC millimole regression and the RC outlier criteria.

Physical Characteristics

A number of physical characteristics were evaluated for their frequency of occurrence in the set of discordant chemicals versus the entire set of reference chemicals. The characteristics chosen were those that were assumed to be available, or relatively easy to measure, for new chemicals that may be tested in these assays to determine starting doses for acute systemic toxicity assays. The characteristics examined included chemical class, molecular weight, boiling point, IC_{50}, pH, and log K_{ow} (i.e., log octanol:water partition coefficient). Unfortunately, these attributes were not available for all chemicals. For example, log K_{ow} was available for only 52 of 72 (72%) chemicals and boiling point was available for only 26

of 72 (36%) chemicals. For boiling points > 200°C, 9 of 13 (69%) chemicals were outliers using the 3T3 NRU test method results and 8 of 13 (62%) chemicals were outliers using the NHK NRU test method results. For molecular weight > 400 g/mole, 5 of 7 (71%) chemicals were outliers using the 3T3 NRU test method results and 3 of 7 (43%) chemicals were outliers using the NHK NRU test method results. For log K_{ow} > 3, 9 of 12 (75%) chemicals were 3T3 outliers and 8 of 12 (67%) chemicals were NHK outliers.

Chemical Class

Examination of outliers by chemical class for the RC regression showed that 3 of 3 (100%) organophosphates were outliers in both *in vitro* NRU test methods.

Solubility

Another attribute that may cause a chemical to be discordant is the lack of solubility in the solvent or when added to the tissue culture media. Since the SMT expected the toxicity of insoluble chemicals to be underpredicted in the *in vitro* NRU cytotoxicity assays, chemical tests with precipitates were noted and compared with the discordant chemicals. However, insolubility was not consistently associated with the discordant chemicals for which toxicity was underpredicted. For example, only 5 of the 24 (21%) underpredicted chemicals identified by applying the 3T3 NRU test method results to the RC regression exhibited signs of insolubility in the 3T3 assay in at least one laboratory (see **Table 5-8** for chemicals that had precipitates in the NRU assays). Additionally, evidence of insolubility was noted for dibutyl phthalate and diethyl phthalate in both assays, but toxicity was overpredicted rather than underpredicted.

For the 3T3 NRU, 25 chemicals showed evidence of insolubility in the 3T3 assay in at least one lab. Eleven (4%) of these chemicals were outliers. For the NHK NRU, 24 chemicals showed evidence of insolubility in at least one laboratory. Nine (38%) of the 24 chemicals were outliers.

Metabolism

The SMT expected that the toxicity of chemicals metabolized *in vivo* to active compounds (see **Table 3-7**) would be underpredicted *in vitro* by 3T3 and NHK cells, which have little or no metabolic capability. Of the 72 reference chemicals used to validate the 3T3 and NHK NRU test methods, 22 (31%) chemicals are known to have active metabolites *in vivo* and only 10 (45%) of these chemicals were classified as outliers for the 3T3 NRU. Of these 10 chemicals, the toxicity of six (60%) chemicals was underpredicted, while the toxicity of four (40%) chemicals was overpredicted. For the NHK NRU, nine (29%) of the 31 outlier chemicals are metabolized to active metabolites. Four (44%) of nine are negative outliers (predicted to be less toxic than they are). See **Table 3-7** for chemicals with active metabolites. Thus, the fact that a chemical has active metabolites does not necessarily indicate that its toxicity will be underpredicted by the *in vitro* NRU cytotoxicity test methods.

Halle (1998) reported similar findings for the RC database (i.e., approximately half of the chemicals metabolized to active metabolites were outliers and half were not).

Mechanism of Action

The contribution of mechanism of action to discordant status was assessed by developing additional regressions for the prediction of LD_{50} values from the *in vitro* NRU IC_{50} values. Both the 3T3 and NHK NRU test method data and the RC database were used for this evaluation. The RC database was used because:

- it is the largest published compilation of IC_{50x} and rodent LD_{50} data
- the RC IC_{50x} data were highly correlated with the 3T3 and NHK NRU IC_{50} data collected for the 58 chemicals in common, and especially for the *in vitro* 3T3 NRU IC_{50} data (see **Figures 5-3** and **5-4**)

In evaluating the contribution of mechanism of action to discordant status based on the RC weight regression, chemicals with mouse LD_{50} data only were excluded from the comparison. RC rat data only were used because:

- rat and mouse data should not be combined, regardless of the high correlation of their LD_{50} data reported in the RC publication on their subset of rat and mouse

LD_{50} values (see **Section 4.1.2**), since rats and mice may not have the same sensitivity to individual chemicals

- the majority of data used in the RC regression were rat data (282 rat data points and 65 mouse data points) (Halle 1998)
- the great majority of acute oral systemic toxicity testing is performed with rats.

The linear regression developed from the 282 rat data points in the RC (see **Appendix K-4**) using weight units is shown in **Table 6-2** and **Figure 6-5**. **Table 6-2** shows that the RC rat weight regression was not significantly different from the weight regression for the complete RC database when slopes and intercepts were simultaneously compared (goodness of fit F test; p=0.961).

The following boxes characterize the 30 discordant chemicals (i.e., outliers) for the 3T3 NRU by counting the number of outliers in each category and comparing to the total number of chemicals in the category.

Physical Form	Number of Outliers/Total in Category
Solid	23 outliers/54 solids
Liquid	7 outliers/18 liquids

Boiling Point (in degrees C)	Outliers/Total
No info	14/34
< 100	1/8
100-200	1/5
200-300	3/4
300-400	5/6
408	1/1
960	0/1
1500	0/1
others decompose, sublime, or BPs are given @ < atmospheric pressure	

Molecular Weight (g/mol)	Outliers/Total in Class
< 150	3/21 chemicals (but no info on MeOH or CCl4)
> 150-200	6/14
200-300	13/20
300-400	3/11
400-500	3/4
500-600	1/1
600-700	0/1
700-800	1/1

Chemical class	Number of Outliers/Total in Class	
Alcohols	3/10	
Carboxylic acids	4/12	
Heterocyclic	7/14	
Mercury compounds	1/1	
Organophosphorous	3/3	(2 were organothiophosphorous cmpds)
Polycyclic	1/2	
Sulfur compound	1/2	
Sodium compound	2/5	
Organometallic	1/1	
Amide	1/3	
Amine	1/1	
Arsenical	1/2	
Boron compounds	0/1	
Cadmium compounds	0/1	
Cyclic hydrocarbon	1/2	
Halogenated hydrocarbon	1/3	
Hydrocarbon	1/2	
Ketone	0/1	
Lithium compound	0/1	
Metal compound	1/1	
Nitrile	0/1	
Phenol	0/1	
Potassium compound	1/2	
Chlorine compound	1/2	
Nitrogen compound	1/1	
Chromium compound	0/1	
Fluorine compound	0/1	
Oxygen compound	1/1	
Selenium compound	1/1	

3T3 IC50 (mM)	Outliers/Total
≤ 0.0001	0/2
0.0001 - 0.001	1/2
0.001 - 0.01	1/4
0.01 - 0.1	9/13
0.1 - 1	15/23
1-10	2/12
10-100	1/9
> 100	1/5

No IC50s for 2 chemicals

3T3 pH	Outliers/Total
< 7.6	0/9
7.6	0/0
7.7	1/1
7.8	0/1
7.9	3/6
8	5/12
8.1	11/18
8.2	3/6
8.3	3/8
8.4	1/5
8.5	0/1
> 8.5	3/4

log Kow	Outliers/Total
< -4	0/1
-4 to –1	2/7
-1 to 0	3/7
0 to 1	4/7
1 to 2	5/13
2 – 3	1/5
3 - 4	5/8
4 - 5	2/2
6 – 7	2/2
No info	6/20

The following boxes characterize the 31 discordant chemicals (i.e., outliers) for the NHK NRU by counting the number of outliers in each category and comparing to the total number of chemicals in the category.

Boiling Point (in degrees C)	Outliers/Total
No info	13/34
< 100	2/8
100-200	1/5
200-300	3/4
300-400	5/6
408	0/1
960	0/1
1500	0/1
others decompose, sublime, or BPs are given @ < atmospheric pressure	

Molecular Weight (g/mol)	Outliers/Total
< 150	5/21 chemicals (but no info on CCl4)
> 150-200	7/14
200-300	13/20
300-400	3/11
400-500	3/4
500-600	0/1
600-700	0/1
700-800	0/1

Chemical class	Outliers/Total	
Alcohols	4/10	
Carboxylic acids	6/12	
Heterocyclic	9/14	
Mercury compounds	1/1	
Organophosphorous	3/3	(2 were organothiophosphorous cmpds)
Polycyclic	0/2	
Sulfur compound	1/2	
Sodium compound	2/5	
Organometallic	0/1	
Amide	0/3	
Amine	1/1	
Arsenical	1/2	
Boron compounds	0/1	
Cadmium compounds	0/1	
Cyclic hydrocarbon	0/2	
Halogenated hydrocarbon	1/3	
Hydrocarbon	1/2	
Ketone	0/1	
Lithium compound	0/1	
Metal compound	0/1	
Nitrile	0/1	
Phenol	0/1	
Potassium compound	1/2	
Chlorine compound	1/2	
Nitrogen compound	1/1	
Chromium compound	0/1	
Fluorine compound	0/1	
Oxygen compound	1/1	
Selenium compound	1/1	

NHK IC50 (mM)	Outliers/Total
≤ 0.0001	0/2
0.0001 - 0.001	1/2
0.001 - 0.01	3/4
0.01 - 0.1	6/13
0.1 - 1	11/23
1-10	8/12
10-100	1/9
> 100	1/5
No IC50s for 2 chemicals	

pH	Outliers/Total
< 7.1	0/6
7.1	0/0
7.2	1/1
7.3	0/0
7.4	1/4
7.5	3/7
7.6	4/7
7.7	9/23
7.8	11/17
7.9	0/3
8	0/1
8.1	0/0
8.2	1/1
8.3	0/0
8.4	0/0
8.5	1/1
> 8.5	0/1

log Kow	Outliers/Total
< -4	0/1
-4 to –1	2/7
-1 to 0	5/7
0 to 1	3/7
1 to 2	5/13
2 – 3	1/5
3 - 4	5/8
4 - 5	2/2
6-7	1/2
No info	7/20

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